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Identification and assessment of TCR-T cells targeting an epitope conserved in SARS-CoV-2 variants for the treatment of COVID-19

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ABSTRACT

Background: Coronavirus disease 2019 (COVID-19) continues to be a major global public health challenge, with the emergence of variants of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Current vaccines or monoclonal antibodies may not well be protect against infection with new SARS-CoV-2 variants. Unlike antibody-based treatment, T cell-based therapies such as TCR-T cells can target epitopes that are highly conserved across different SARS-CoV-2 variants. Reportedly, T cell-based immunity alone can restrict SARS-CoV-2 replication.

Methods: In this study, we identified two TCRs targeting the RNA-dependent RNA polymerase (RdRp) protein in CD8⁺ T cells. Functional evaluation by transducing these TCRs into CD8⁺ or CD4⁺ T cells confirmed their specificity.

Results: Combinations of inflammatory and anti-inflammatory cytokines secreted by CD8⁺ and CD4⁺ T cells can help control COVID-19 in patients. Moreover, the targeted epitope is highly conserved in all emerged SARS-CoV-2 variants, including the Omicron. It is also conserved in the seven coronaviruses that infect humans and more broadly in the subfamily Coronavirinae.

Conclusions: The pan-genera coverage of mutant epitopes from the Coronavirinae subfamily by the two TCRs highlights the unique strengths of TCR-T cell therapies in controlling the ongoing pandemic and in preparing for the next coronavirus outbreak.

1. Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection and the caused coronavirus disease-2019 (COVID-19) are still a global health challenge. Mass administrations of vaccines and the use of COVID-19 approved antiviral drugs like Molnupiravir, and monoclonal antibodies have reduced the rate of severe and fatal cases [1,2]. However, sustained efforts are still required to deal with the rapidly emerging SARS-CoV-2 variants. Compared with other single-stranded RNA viruses, the estimated mutation rates of coronaviruses are moderate to high and can generate novel variants resistant to current vaccines and treatments [3–5], making people susceptible to infection and

disease after being exposed to them [6,7]. In addition, most current vaccines mainly elicit immunity against the Spike protein which is rapidly mutating during virus circulation. In contrast, the viral polymerase is one of the putative targets for T cell responses with pan-Coronaviridae reactivity, because it is likely to be highly conserved due to their key early roles in the viral life cycle.

The majority of those who recover from COVID-19 exhibit robust and broad SARS-CoV-2 specific T cell responses [8]. Lower levels of CD8⁺ T cells and dendritic cells (DCs) but higher levels of macrophages and neutrophils have been found in patients with severe disease. There was a lesser clonal expansion of CD8⁺ T cells from patients with severe disease compared with those with moderate disease, implicating a compromised

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SARS-CoV-2 specific CD8⁺ T cell response in severe cases [9]. In general, SARS-CoV-2 specific CD8⁺ T cells can help prevent severe clinical symptoms [10], while specific CD4⁺ T cells are positively correlated with the level of neutralizing antibodies [11]. Sufficient T cell clonal expansion has been recorded in COVID-19 convalescent patients, highlighting the importance of SARS-CoV-2 specific T cell-mediated control of viral replication [12].

TCR-transduced T (TCR-T) cells against several tumor antigens including MART1, CEA, gp100, NY-ESO-1, and MAGEA3, have been tested in clinical trials, and have shown great promise for the treatment of various types of cancers [13,14]. Unlike antibody-based treatment, T cell-based therapies such as TCR-T cells can target epitopes that are highly conserved across different SARS-CoV-2 variants, as well as other coronaviruses strains. The development of TCR-T therapy can help defend against upcoming variants of SARS-CoV-2 [15] and prepare for the next coronavirus outbreak [16]. TCR-T cells active against SARS-CoV-2 variants can be administered even before infection to allow the establishment of virus-specific memory T cells. They can be useful for the protection of healthcare workers and high-risk patients in a prophylactic setting. This therapy also needs to be provided to immunodeficient patients who do not mount a sufficient immune response following vaccination. Moreover, compared with the many years required for the *de novo* development of effective drugs for newly emerged coronaviruses, the identification of effective TCRs and generation of TCR-T cells requires a much shorter period of time (e.g., several months).

In this study, we identified and evaluated two SARS-CoV-2 specific TCRs targeting a highly conserved epitope derived from RNA-dependent RNA polymerase (RdRp). The targeted epitope is highly conserved among different SARS-CoV-2 variants, including Omicron. It is also conserved in the seven coronaviruses that infect humans and more broadly in the subfamily *Coronavirinae*. The pan-genera coverage of mutant epitopes from the *Coronavirinae* subfamily by the two TCRs highlights the potential value of TCR-T therapies in controlling the ongoing pandemic.

2. Materials and Methods

2.1. Isolation of PBMC

The PBMC were isolated from buffy coats obtained from healthy donors who had signed informed consent by density gradient centrifugation using Lymphoprep (Dakewe, DKW-LSH-0250). The freshly isolated PBMC were cryopreserved by dissolving in cell banker 2 (ZENOAQ). This study was approved (20201130003-FS01) by the institutional review board of the Second People's Hospital of Shenzhen, Shenzhen, China.

2.2. Peptides

The 9-mer RdRp₈₂₉₋₈₃₇ peptide (LPYPDPSRI), 10-mer RdRp₈₂₉₋₈₃₈ peptide (LPYPDPSRIL) and the four RdRp₈₂₉₋₈₃₇ variant peptides ((LPYPNPSRI, LPYPDASRI, LPYPDVSRI, and YPYDPVSRI) (purity > 95 %) were synthesized, purified by high-performance liquid chromatography and validated by mass spectrometry from Genscript (China).

2.3. T cell stimulation and expansion of epitope-specific T cells

Pre-existing T cells targeting a highly conserved epitope from RdRp protein have been identified in previous study [17]. The epitope-specific T cells were then sorted and expanded as follows. PBMCs depleted of CD4⁺T cells (3×10^6) were stimulated by RdRp₈₂₉₋₈₃₈ peptide (10 μM) for 8 days at 37 °C. Recombinant human IL-2 (PEPROTECH, 200–02) at 50 IU/ml was added on day 1. Cells were harvested 8 days post stimulation. The CD8⁺ 4-1BB⁺ T cells were sorted by fluorescence-activated cell sorting (FACS) after overnight stimulation by the corresponding

peptide and expanded by a T cell rapid expansion protocol (REP) as described in [18]. The function of expanded T cells was evaluated by incubation with RdRp₈₂₉₋₈₃₈ or RdRp₈₂₉₋₈₃₇ (10 μM). The CD8⁺ 4-1BB⁺ T cells were sorted again for Next Generation Sequencing (NGS)-based TCR sequencing.

2.4. Flow cytometry

T cells were analyzed by flow cytometry (FCM). Briefly, cells were suspended in FCM buffer of sterile phosphate buffered saline (PBS, Corning, 21-040-CV) plus 1 % bovine serum albumin (Roche, 10735078001). T cells were then stained by: PE-Cy7-mTRBC (clone H57-597, BD Biosciences, 560729), APC-CD8 (clone RPA-T8, BD Biosciences, 555369) or FITC-CD8 (clone RPA-T8, BD Biosciences, 555366), Alexa Fluor 700-CD4 (clone SK3, BD Biosciences, 566318), BV421-4-1BB (clone 4B4-1, BD Biosciences, 564091), PE-OX40 (clone ACT35, BD Biosciences, 555838), APC-CD107a (clone H4A3, BD Biosciences, 560664), PE-IFN-γ (Miltenyi Biotec, 130-054-202) and 7-AAD (BD Biosciences, 559925) for removal of dead cells. The stained cells were washed and re-suspended in FCM buffer and analyzed using a FACS Aria II (BD Biosciences, San Jose, CA).

2.5. T cell repertoire preparation, high throughput sequencing and data analysis

T cell repertoire preparation, high throughput sequencing and data analysis were performed by GENEWIZ (Guangzhou, China). Briefly, total RNA was extracted from the CD8⁺ 4-1BB⁺ T cells sorted from the REP cultures using Trizol (Invitrogen, 15596018) according to the user manual. 5' RACE was performed with SMARTer RACE cDNA Amplification Kit (Clontech, 634859), total RNA input was 1 μg. TRAV and TRBV NGS libraries were made by using NEBNext Ultra DNA Library Prep Kit for Illumina (NEB). After quality control on a Bioanalyzer High Sensitivity DNA chip (Agilent), libraries were sequenced on the Illumina Miseq 2 × 300 platform. Trimmomatic software was adopted to finish QC for raw reads with default parameters. After QC, reads with adapters will be discarded and the bases in reads with quality lower than 20 will be also removed. The clean reads were processed by MIXCR software to identify clones and corresponding CDR sequences. In this processing, MIXCR software utilizes V, D, J gene reference sequences from T cell. After the clones and CDR sequences information were obtained, customized scripts were written to do the following statistics.

2.6. Retroviral transduction

TCR-T cells were generated by using PBMC from one healthy donor and cultured as previously described [19]. Briefly, TCR variable chains were codon-optimized and synthesized by Sangon Biotech (Shanghai, China). Both TCR α and β constant chains were substituted with the corresponding murine constant chains. The TCR α and β variable chains were fused with the mouse α and β constant chains respectively. The hybrid TRA and TRB chains were then fused by a furin plus P2A element and cloned into the retroviral vector MSGV1 (Purchased from Addgene, #107227). 293 T cells were used for transfection of retrovirus plasmids: TCR plasmid, VSV-G and Gag-Pol by Lipofectamine 2000 (Invitrogen, 11668019) according to the manufacturer's protocol. Retrovirus supernatant was centrifuged at 2000g for 2 h in non-tissue culture plates pre-coated with 20 μg/mL RetroNectin (Takara, T100A). Then the retrovirus supernatant was removed and pre-stimulated PBMCs were added and cultured overnight in 5 % CO₂ incubator at 37 °C.

2.7. Co-culture with target cells

The catalytic unit of RdRp protein (nsp12) of the SARS-CoV-2 Wuhan-Hu-1 strain were obtained from Genbank (NCBI Reference Sequence:NC_045512.2). COS-7 cells stably transfected with HLA-

A*B51:01, RdRp-EGFP or both were generated for co-culture assay. TCR-T cells (5×10^4 – 1×10^5) were incubated with peptide-loaded COS-7-B51:01 or wildtype control (COS-7- wt). COS-7-B*51:01 or COS-7- wt cells (5×10^4 – 1×10^5) were loaded with 10 μ M corresponding peptides or a serial of dilution of peptides. Regarding to the evaluation of functional affinity of RdRp₈₂₉₋₈₃₇ variant peptides, TCR-T cells (5×10^4 cells) were incubated overnight with COS-7-B*51:01 cells loaded with a serial dilution of the four RdRp₈₂₉₋₈₃₇ variant peptides (LPYPNPSRI, LPYPDASRI, LPYPDVSRI, and YYPDPVSRI). After 16–24 h co-culture with T cells, supernatants were taken and analyzed for IFN- γ by ELISA. When measuring the recognition of intracellular epitope, TCR-T cells were directly incubated with COS-7-B*51:01-RdRp cells or control target cells at a ratio of 1:1 without loading of peptides. The anti-HLA-B/C antibody (clone B1.23.2, Invitrogen) was added at a final concentration of 10 μ g/mL when needed.

2.8. IFN- γ ELISA assay

An anti-IFN- γ monoclonal antibody has been used as primary antibody (Clone 2G1, Invitrogen, M700A) which was coated on the MaxiSorp 96-well plate (Nunc, 442404) overnight at 4 °C. Antibody was removed, and plates were blocked by PBS plus 1 % BSA for 2 h. Cells were co-cultured as previously mentioned, and supernatants were transferred into pre-coated plates for incubation. A biotin labeled anti-IFN- γ antibody has been used as a secondary antibody (Clone B133.5, M701B, Invitrogen), and HRP conjugated streptavidin (Thermo Scientific, N200) was added after secondary antibody incubation. 100 μ L/well TMB (Sigma Aldrich, T5525) has been added as substrate of HRP. Plates were read by infinite M200 PRO reader (TECAN Austria GmbH, Grödlg, Austria). OD₄₅₀ was used for the readout and OD₆₃₀ was used as reference readout for background control. Technical replicates have been performed for ELISA assay.

2.9. IFN- γ ELISPOT assay

ELISPOT assay was performed as described previously [17]. Briefly, Primary antibody was coated on 96-well PVDF plates (Millipore, MSIPS4510) overnight at 4 °C. The plates were washed six times with PBS to remove unbound primary antibody. TCR-T cells mixed with corresponding target cells (with peptides or not) were seeded. After incubation for 18–20 h in 5 % CO₂ incubator at 37 °C, plates were washed with PBS. Biotinylated anti-human IFN- γ was added and incubated for 2 h at room temperature. After incubation, plates were washed and incubated further for 1 h with streptavidin-conjugated HRP. Next, the plates were washed as before and 100 μ L of AEC (Sigma-Aldrich, AEC101) were added to the wells. Finally, plates were washed under running tap water and dried at room temperature. IFN- γ spot-forming cells (SFC) were enumerated using an ELISPOT reader (C.T.L., Shaker Heights, OH).

2.10. Meso Scale discovery (MSD) measurement

Supernatants were taken after overnight co-culture of TCR-T cells with COS-7-B*51:01-RdRp cells, and analyzed by the MSD kit (Meso Scale Diagnostics, Rockville, MD, USA) following the manufacturer's instructions. Cytokine levels were determined using a MESOTM QuickPlex SQ 120 (Meso Scale Diagnostics, Rockville, MD, USA).

2.11. Statistical analysis

Statistical analysis was performed using unpaired Student's *t* test. Statistical significance was set at $p < 0.05$. The data are shown as mean value (\pm standard deviation).

3. Results

3.1. Identification of SARS-CoV-2 specific TCRs targeting a highly conserved epitope from RdRp protein

SARS-CoV-2 specific T cells targeting a highly conserved epitope derived from the RdRp protein (RdRp₈₂₉₋₈₃₈, LPYPDPSRI) were identified from several healthy donors in our previous study, and this epitope was predicted to be HLA-B*51:01 restricted by NetMHCpan4.1 [17,20]. CD8⁺4-1BB⁺ T cells were sorted from one pre-pandemic healthy donor for expansion followed by activity verification. Meanwhile, a truncated epitope with a higher predicted binding potential to B*51:01 (RdRp₈₂₉₋₈₃₇, LPYPDPSRI) was selected and experimentally verified (Supplementary Fig. 1A and 1B) [20]. CD8⁺4-1BB⁺ T cells from the expanded cultures were sorted for NGS-based TCR identification, as previously reported [19]. Two dominant TRBVs with two different CDR3s and three dominant TRAVs with three different CDR3s were identified (Fig. 1A). Six different TCRs were assembled (TRAV38-2 with TRBV7-6, TRAV23 with TRBV7-6, TRAV24 with TRBV7-6, TRAV38-2 with TRBV7-9, TRAV23 with TRBV7-9, TRAV24 with TRBV7-9) and transduced into autologous PBMCs for functional evaluation. As shown in the ELISA assay (Fig. 1B), the combination of TRAV38-2 with TRBV7-6 and TRAV24 with TRBV7-9 showed specific recognition of both the epitopes RdRp₈₂₉₋₈₃₇ and RdRp₈₂₉₋₈₃₈. We further verified TCR-HLA-dependent recognition using FACS analysis of the T cell activation marker 4-1BB. As shown in Fig. 1C, the population of CD8⁺4-1BB⁺ and CD4⁺4-1BB⁺ T cells showed specific increases in both combinations. In particular, the epitope RdRp₈₂₉₋₈₃₇ induced a higher activation than RdRp₈₂₉₋₈₃₈ in both of ELISA and FACS assays for the combination of TRAV38-2 and TRBV7-6. For TRAV24 with TRBV7-9, CD4⁺ T cells showed significantly higher potency with RdRp₈₂₉₋₈₃₇ than RdRp₈₂₉₋₈₃₈ (Fig. 1C). Therefore, the epitope RdRp₈₂₉₋₈₃₇ was used in subsequent studies. We renamed the two functional TRAV and TRBV combinations as TCR1 and TCR2 (Fig. 1D) and generated the TCR-T cells by transducing them to non-self PBMCs for subsequent studies. Furthermore, the six HLA-I alleles (HLA-A*02:03, A*33:03, B*38:02, B*51:01, C*07:02, and C*14:02) of the original healthy donor were expressed in COS-7 cells for functional evaluation. Both TCR1-T and TCR2-T cells showed specific recognition of RdRp₈₂₉₋₈₃₇ when loaded on HLA-B*51:01 only (Fig. 1E).

3.2. Functional evaluation of CD8⁺TCR-T cells

Because the two TCRs were identified from CD8⁺ T cells, we generated CD8⁺TCR1 and CD8⁺TCR2-T cells for further functional evaluation. Both TCRs recognized the RdRp₈₂₉₋₈₃₇ epitope present on HLA-B*51:01 molecules (Fig. 2A). Neither showed recognition for the nucleocapsid protein 67–75 epitope (N₆₇₋₇₅, FPRGQGVPI) that is also reported to be HLA-B*51:01 restricted (IEDB database [21]). FACS analysis showed that activation markers (4-1BB and OX40) of T cells transduced with TCRs (mouse TRB constant region positive [mTRBC⁺]) were also upregulated (Fig. 2B, C). We obtained similar results in the ELISPOT assay (Fig. 2D)—both TCR1-CD8⁺ and TCR2-CD8⁺ T cells showed specific recognition for RdRp₈₂₉₋₈₃₇, whereas CD8⁺Con-T cells (Control T cells) did not. The functional avidity of the CD8⁺TCR-T cells was evaluated using a peptide dilution assay (Fig. 2E). Both CD8⁺TCR1 and CD8⁺TCR2-T cells showed concentration-dependent recognition of the RdRp₈₂₉₋₈₃₇ epitope, and neither showed recognition of the N₆₇₋₇₅ epitope.

3.3. Functional evaluation of CD4⁺ TCR-T cells

One of the advantages of generating TCR-T cells is that, unlike the epitope-induced SARS-CoV-2 specific T cells, the TCRs can be transduced to both CD8⁺ and CD4⁺ T cells. CD4⁺ T cells are also critical for proper disease control of COVID-19 [11]. Hence, we also generated CD4⁺ TCR1 and CD4⁺ TCR2-T cells for functional evaluation. Like CD8⁺

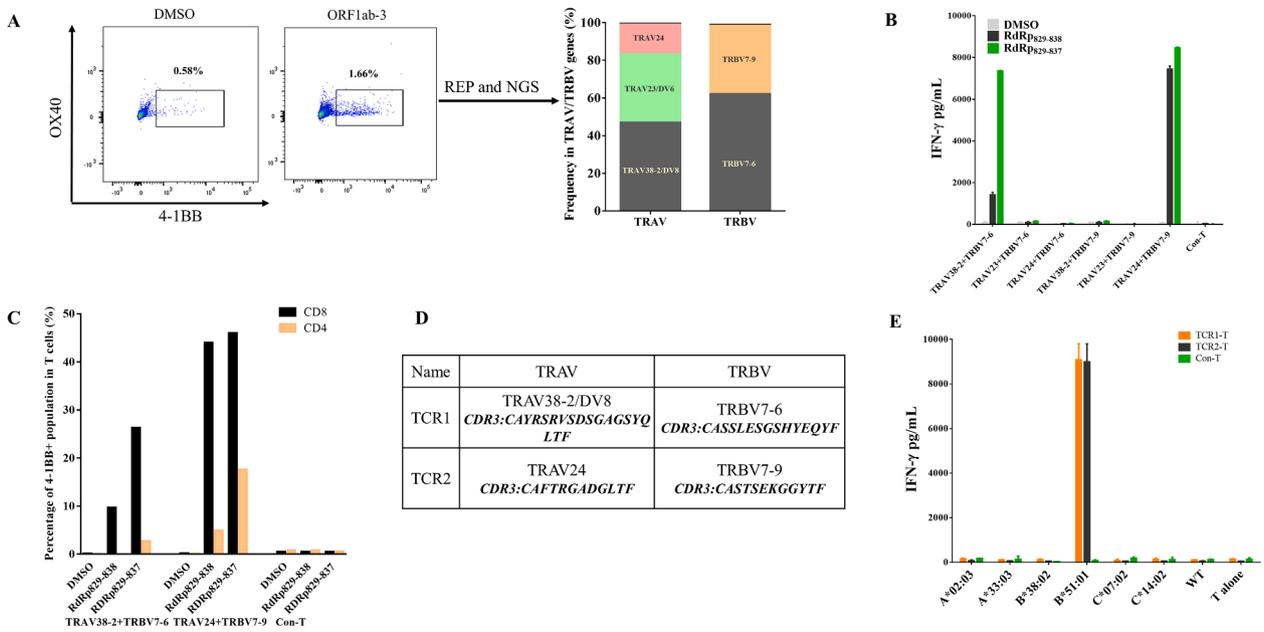


Fig. 1. Next Generation Sequencing-based TCR identification from one pre-pandemic healthy donor and followed functional evaluation. (A). CD8⁺4-1BB⁺T cells were sorted and expanded by rapid expansion protocol (REP). The activity of expanded cells was verified by measuring of IFN- γ release in ELISA upon peptide (RdRp₈₂₉₋₈₃₈ epitope) stimulation. The CD8⁺ 4-1BB⁺ T cells were again sorted (around 8×10^5) for NGS by fluorescence-activated cell sorting (FACS) from REP cultures after overnight stimulation by peptide stimulation (RdRp₈₂₉₋₈₃₈ epitope). (B). TCRs with 6 different TRAV and TRBV combinations were assembled and transduced to autologous PBMCs. Supernatants were taken and IFN- γ were measured in ELISA assay after overnight stimulation by the corresponding peptides or the solvent control (DMSO). IFN- γ production depicted as mean values of three replicates (mean \pm SD). (C). FACS analysis of T cell activation marker 4-1BB was performed after overnight stimulation by the corresponding peptides or DMSO. (D) The two functional TRAV and TRBV combinations were renamed as TCR1 and TCR2 respectively. (E). IFN- γ measurement by ELISA after overnight co-incubation of TCR1-T cells and TCR2-T cells with RdRp₈₂₉₋₈₃₇ epitope loaded COS-7 cells expressing the 6 HLA-I alleles of the originate health donor. IFN- γ production depicted as mean values of three replicates (mean \pm SD).

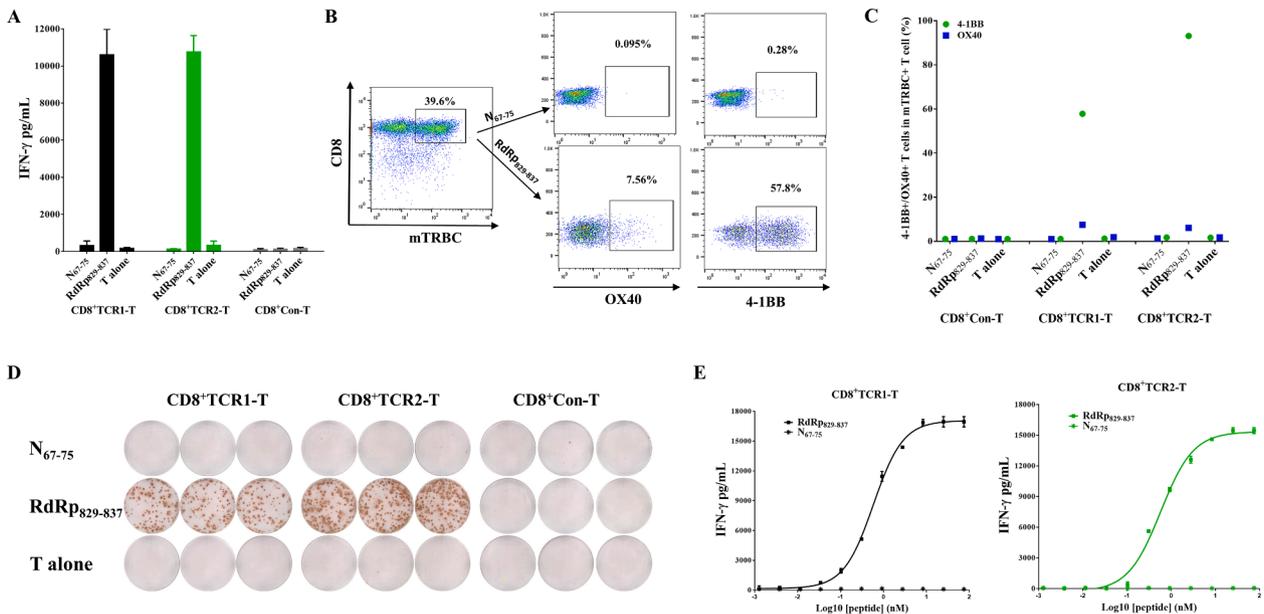


Fig. 2. Functional evaluation of CD8⁺TCR-T cells. (A). IFN- γ measurement by ELISA after overnight co-incubation of CD8⁺TCR1-T, CD8⁺TCR2-T and CD8⁺Con-T cells with COS-7-B*51:01 cells loaded with RdRp₈₂₉₋₈₃₇ or the control epitope (N67-75). IFN- γ production depicted as mean values of three replicates (mean \pm SD). (B) and (C). FACS analysis of T cell activation markers of 4-1BB and OX40 after overnight co-cultures. (D). ELISPOT detection of CD8⁺TCR1-T, CD8⁺TCR2-T and CD8⁺Con-T cells co-incubating with COS-7-B*51:01 cells loaded with RdRp₈₂₉₋₈₃₇ or N67-75. (E). COS-7-B*51:01 cells loaded with a serial dilution of different epitopes were incubated with CD8⁺TCR1-T and CD8⁺TCR2-T cells overnight. The supernatants were taken for quantification of IFN- γ in ELISA assay. IFN- γ production depicted as mean values of three replicates (mean \pm SD).

TCR-T cells, both CD4⁺ TCR1 and CD4⁺ TCR2-T cells transduced with TCRs (mTRBC⁺) showed specific recognition of RdRp₈₂₉₋₈₃₇ epitopes loaded onto B*51:01 (Fig. 3A). Moreover, this recognition was

significantly blocked by an anti-HLA-B/C antibody (clone B1.23.2) ($p < 0.05$). FACS analysis showed that T cell activation markers 4-1BB and OX40 were upregulated (Fig. 3B, C). Same as ELISA results, both the

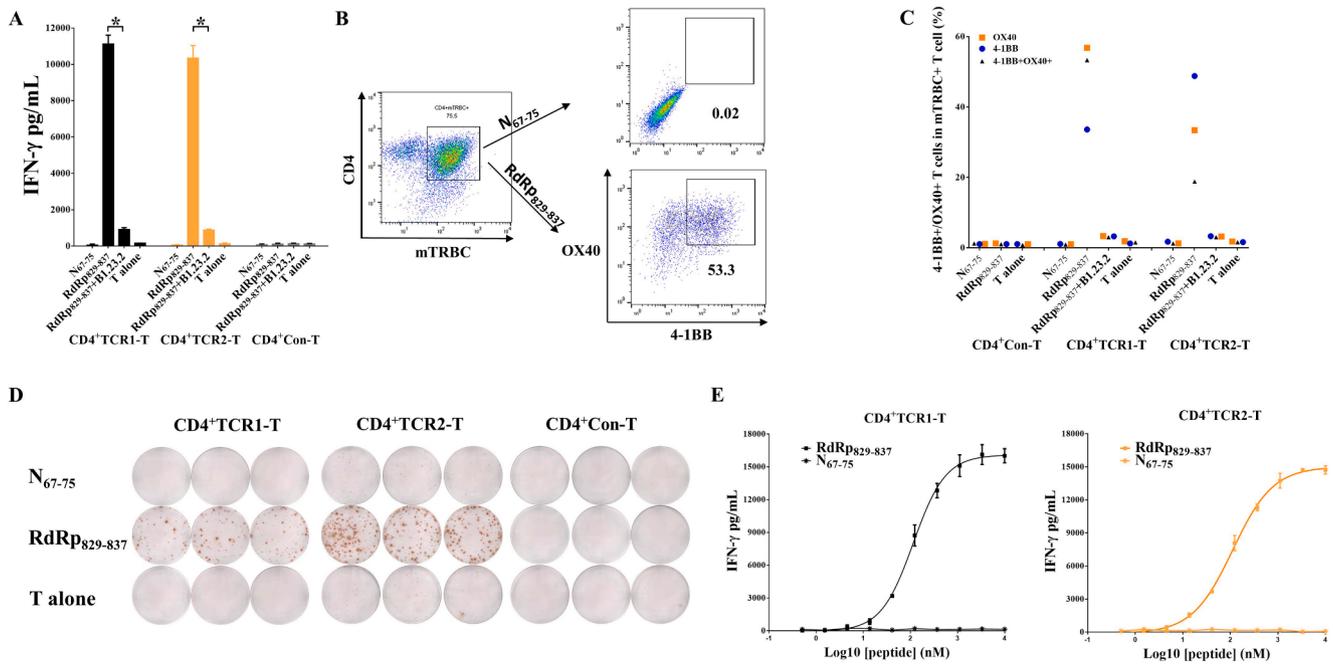


Fig. 3. Functional evaluation of CD4⁺TCR-T cells. (A). IFN-γ measurement by ELISA after overnight co-incubation of CD4⁺TCR1-T, CD4⁺TCR2-T and CD4⁺Con-T cells with COS-7-B*51:01 cells loaded with RdRp829-837 or N67-75 with or without the adding of anti-HLA-B/C antibody (clone B1.23.2). IFN-γ production depicted as mean values of three replicates (mean ± SD). **p* < 0.05 (B) and (C). FACS analysis of T cell activation markers of 4-1BB and OX40 after overnight co-cultures. (D). ELISPOT detection of CD4⁺TCR1-T, CD4⁺TCR2-T and CD4⁺Con-T cells co-incubating with COS-7-B*51:01 cells loaded with RdRp829-837 or N67-75. (E). COS-7-B*51:01 cells loaded with a serial dilution of different epitopes were incubated with CD4⁺TCR1-T and CD4⁺TCR2-T cells overnight. The supernatants were taken for quantification of IFN-γ in ELISA assay. IFN-γ production depicted as mean values of three replicates (mean ± SD).

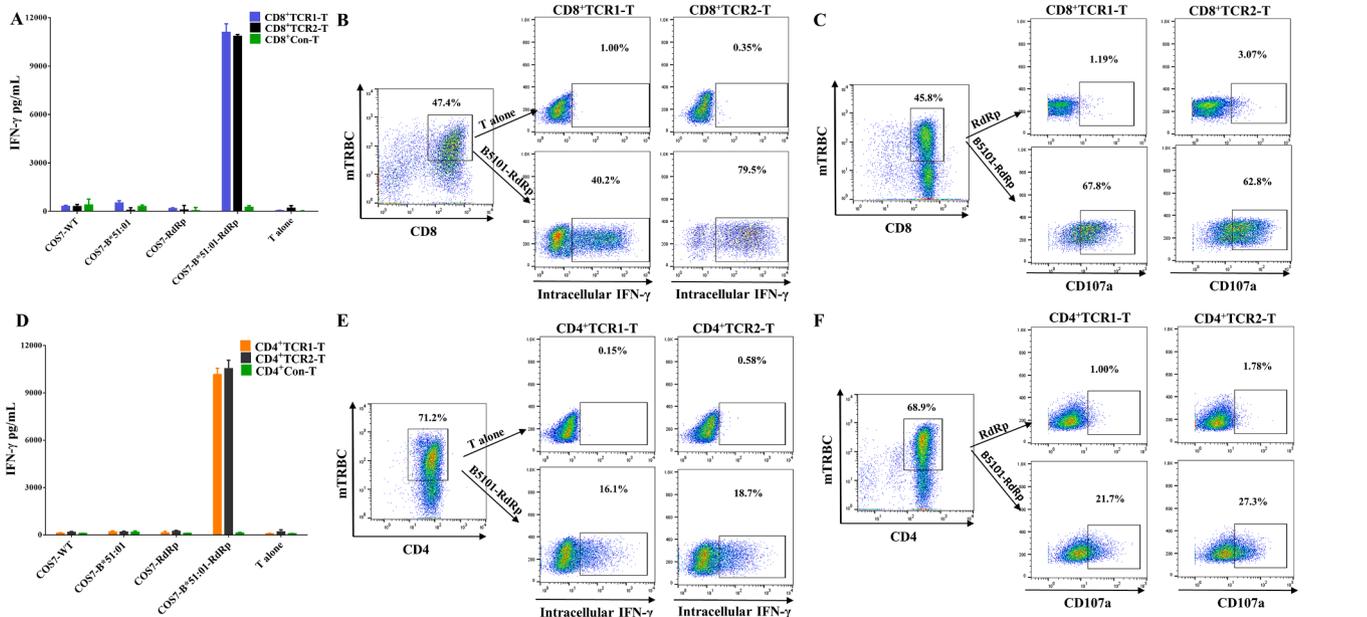


Fig. 4. Functional recognition of intracellular presented epitope. (A). IFN-γ measurement by ELISA after overnight co-incubation of CD8⁺TCR1-T, CD8⁺TCR2-T and CD8⁺Con-T cells with COS-7-B*51:01-RdRp cells, COS-7-B*51:01 cells and COS-7-RdRp cells respectively. IFN-γ production depicted as mean values of three replicates (mean ± SD). (B). FACS analysis of intracellular IFN-γ after overnight co-incubation of CD8⁺TCR1-T, CD8⁺TCR2-T and CD8⁺Con-T cells with COS-7-B*51:01-RdRp cells. (C). FACS analysis of CD107a expression after overnight co-incubation of CD8⁺TCR1-T, CD8⁺TCR2-T and CD8⁺Con-T cells with COS-7-B*51:01-RdRp cells and COS-7-RdRp cells respectively. (D). IFN-γ measurement by ELISA after overnight co-incubation of CD4⁺TCR1-T, CD4⁺TCR2-T and CD4⁺Con-T cells with COS-7-B*51:01-RdRp cells, COS-7-B*51:01 cells and COS-7-RdRp cells respectively. IFN-γ production depicted as mean values of three replicates (mean ± SD). (E). FACS analysis of intracellular IFN-γ after overnight co-incubation of CD4⁺TCR1-T, CD4⁺TCR2-T and CD4⁺Con-T cells with COS-7-B*51:01-RdRp cells. (F). FACS analysis of CD107a expression after overnight co-incubation of CD4⁺TCR1-T, CD4⁺TCR2-T and CD4⁺Con-T cells with COS-7-B*51:01-RdRp cells and COS-7-RdRp cells respectively.

upregulation of 4-1BB and OX40 can also be blocked by the anti-HLA-B/C antibody (Fig. 3C). Similar results were obtained using ELISpot assay. Both CD4⁺ TCR1 and CD4⁺ TCR2-T cells showed specific recognition of RdRp₈₂₉₋₈₃₇, while CD4⁺ Con-T cells did not (Fig. 3D). Moreover, like CD8⁺ TCR-T cells, both CD4⁺ TCR1 and CD4⁺ TCR2-T cells showed a concentration-dependent recognition of the epitope in the peptide dilution assay. Neither showed recognition of the N₆₇₋₇₅ epitope.

3.4. Functional recognition of intracellularly presented epitope

When viruses replicate inside cells or antigen-presenting cells (APC) phagocyte virus-derived antigens [22], the RdRp proteins would be intracellularly processed into their epitopes. Therefore, it is important to verify the recognition of RdRp epitopes present intracellularly. Both CD8⁺ TCR1 and CD8⁺ TCR2-T cells can specifically recognize COS-7-B*51:01-RdRp but are not activated by COS-7-RdRp or COS-7-B*51:01 cells (Fig. 4A). CD8⁺ Con-T cells did not exhibit non-specific recognition. FACS analysis verified upregulation of the T cell activation marker 4-1BB (Supplementary Fig. 1C). Intracellular staining of released IFN- γ showed that mTRBC⁺CD8⁺ T cells can specifically recognize COS-7-B*51:01-RdRp (Fig. 4B), whereas Con-T cells do not (data not shown). Moreover, the potential cytotoxic activity of both CD8⁺ TCR1 and CD8⁺ TCR2-T cells was evaluated by measuring CD107a expression [23]. As shown in Fig. 4C, the TCR⁺CD107a⁺ population of both CD8⁺ TCR1 and CD8⁺ TCR2-T cells showed a specific potent increase when co-incubated with COS-7-B*51:01-RdRp, but not COS-7-RdRp cells.

Similarly, we also evaluated the functional recognition of intracellular epitopes by CD4⁺ TCR-T cells. Both CD4⁺ TCR1 and CD4⁺ TCR2-T cells can specifically recognize COS-7-B*51:01-RdRp (Fig. 4D), which was also verified by the upregulated expression of 4-1BB and OX40 in FACS analysis (Supplementary Fig. 1D). Moreover, the proportion of CD4⁺mTRBC⁺IFN- γ ⁺T cells was significantly upregulated after co-culture with COS-7-B*51:01-RdRp cells (Fig. 4E). Interestingly, the upregulation of CD107a was also detected in both CD4⁺ TCR1 and CD4⁺ TCR2-T cells (Fig. 4F).

3.5. The profile of functional cytokines of CD8⁺ and CD4⁺TCR-T cells

The secreted cytokines, including IFN- γ , IL-10, IL-12, IL-13, IL-1 β , IL-2, IL-4, IL-6, and IL-8 were quantified using MSD. Upon overnight co-culturing with COS-7-B*51:01-RdRp cells, CD8⁺ TCR-T cells can secrete a panel of inflammatory cytokines, including IFN- γ , IL-13, IL-2, IL-8, and TNF- α (Fig. 5A). Moreover, CD4⁺ TCR-T cells released a panel of inflammatory cytokines, including IFN- γ and IL-8, as well as the anti-inflammatory cytokines IL-13 and IL-10 (Fig. 5B). The level of the cytotoxic cytokine IFN- γ secreted by CD8⁺ TCR-T cells was five times

higher than that of CD4⁺ TCR-T cells. No nonspecific recognition was observed for CD8⁺ and CD4⁺ Con-T cells.

3.6. Conservation of the RdRp₈₂₉₋₈₃₇ epitope

SARS-CoV-2 and its variants belong to the subgenera Sarbecovirus, same as SARS-CoV-1 (Fig. 6A). Protein alignment of the latest strains from different SARS-CoV-2 variants (alpha, beta, gamma, epsilon, eta, Iota, kappa, mu, zeta, delta, and omicron) revealed that this epitope is strictly conserved in Sarbecovirus (Fig. 6B). More broadly, the seven coronaviruses that infect humans belong to the subgenera Embecovirus, Merbecovirus, Sarbecovirus, and the genus Alphacoronavirus (Fig. 6A). Protein alignment revealed that the RdRp₈₂₉₋₈₃₇ epitope was conserved in six of them (6/7) with a mutant epitope (LPYPNP $\underline{\text{S}}$ RI) that emerged in HCoV-OC43 (Fig. 6C). Protein alignment of reference sequences from the four genera of the *Coronavirinae* subfamily found that the epitope was conserved in 44 of 52 strains. In total, four mutant epitopes were identified, including those identified from HCoV-OC43. The other three mutant epitopes were LPYPD $\underline{\text{A}}$ SRI, LPYPD $\underline{\text{V}}$ SRI, and YYPD $\underline{\text{V}}$ SRI (Fig. 6D). We performed functional evaluations to determine whether the two TCRs could recognize these four mutant epitopes. Both TCRs showed similar functional avidity for the mutant epitope LPYPD $\underline{\text{A}}$ SRI (Fig. 6E). While CD8⁺ TCR1-T cells were clearly much more potent for LPYPD $\underline{\text{V}}$ SRI (Fig. 6G). Moreover, both TCRs showed similar functional avidity for the mutant epitope YYPD $\underline{\text{V}}$ SRI, which is less potent than LPYPD $\underline{\text{A}}$ SRI (Fig. 6H).

4. Discussion

COVID-19 continues to be a major global public health challenge due to the sustained emergence of SARS-CoV-2 variants. Developing treatments capable of covering current and upcoming variants is important to control the current pandemic. In this study, we identified two TCRs targeting the highly conserved epitope derived from the RdRp protein. The functional evaluation highlights their potential value in the treatment of COVID-19 infections by current and upcoming SARS-CoV-2 variants.

RdRp₈₂₉₋₈₃₇ reactive polyclonal T cells were expanded and sequenced using the bulk sequencing approach-NGS. As we reported previously, NGS-based TCR identification is an efficient, labor-saving, and cost-effective approach [19]. TRBVs were dominated by TRBV7-6 and TRBV7-9. We identified two functional TCRs using an α - β chain combination-based pairing strategy. The scanning of HLA-I alleles confirmed HLA restriction of the epitope by HLA-B*51:01, consistent with the previous report [17], and anti-HLA-B/C antibodies blocked the

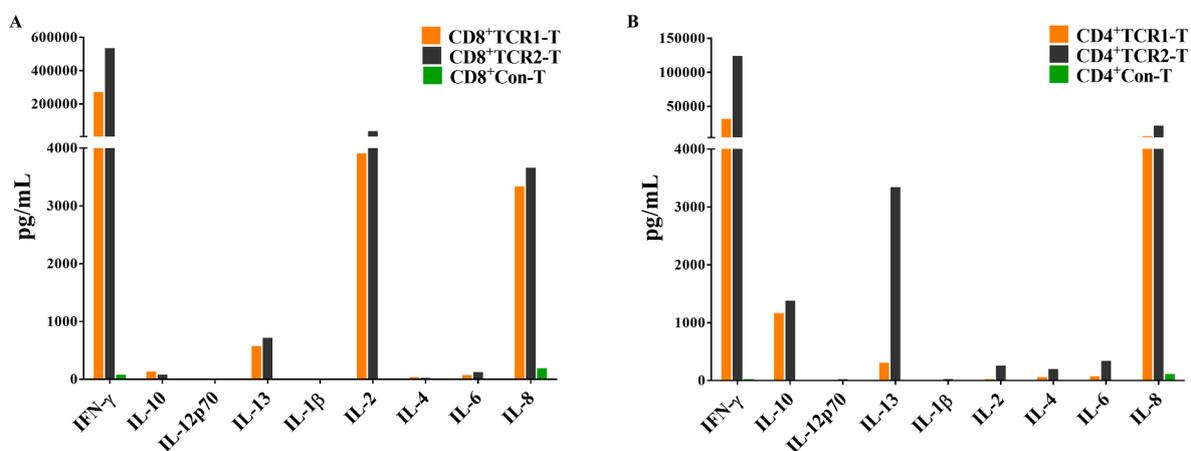


Fig. 5. The profile of functional cytokines of CD8⁺ and CD4⁺TCR-T cells. Supernatants were taken after overnight co-cultures and were analyzed by the MSD. (A). The cytokines secreted by CD8⁺TCR-T cells. (B). The cytokines secreted by CD4⁺TCR-T cells.

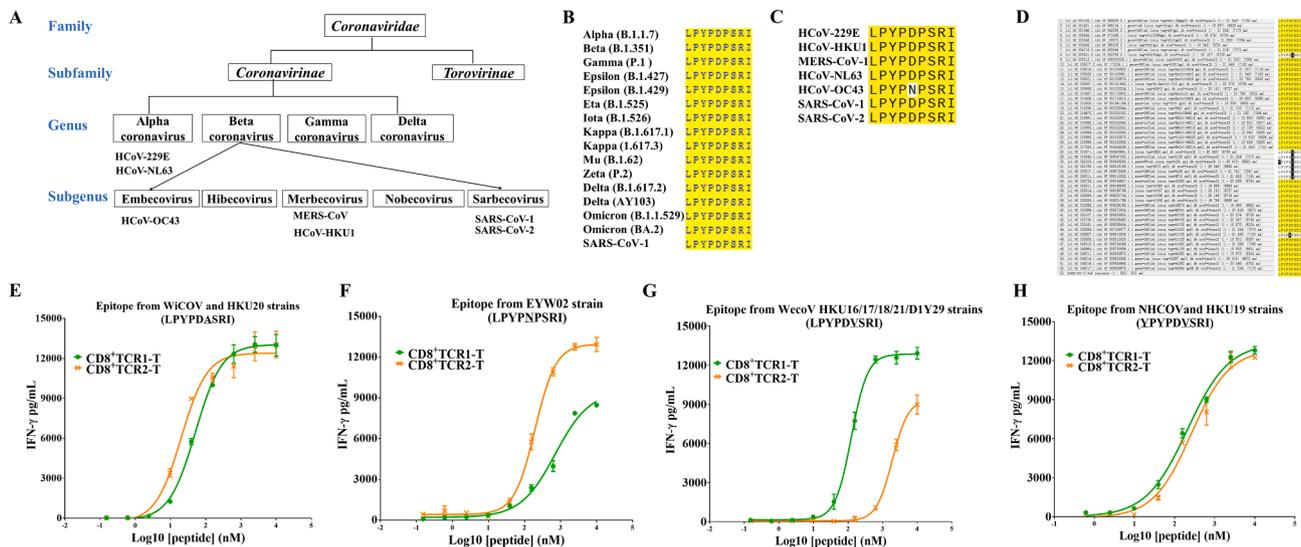


Fig. 6. Conservation of the RdRp₈₂₉₋₈₃₇ epitope. (A). The classification of the *Coronavirinae* subfamily. (B). Protein alignment of Sarbecovirus including SARS-CoV-2 variants and SARS-CoV-1. (C). Protein alignment of the 7 coronaviruses that infect humans. (D). Protein alignment of reference sequences from the four genera of *Coronavirinae* subfamily. (E)(F)(G)(H). COS-7-B*51:01 cells were respectively loaded with a serial dilution of the mutant epitope LPYPDASRI, LPYPNSRI, LPYPDVSRI, and YPYDVSRI. The supernatants were taken for quantification of IFN- γ in ELISA assay after overnight co-incubation with CD8⁺TCR1-T and CD8⁺TCR2-T cells. IFN- γ production depicted as mean values of three replicates (mean \pm SD).** (A). Functional evaluation of the polyclonal T cells after REP. Functions were evaluated by IFN- γ measurement in ELISA after overnight stimulation by the corresponding peptides or DMSO with or without the adding of anti-HLA-B/C antibody (clone B1.23.2). IFN- γ production depicted as mean values of three replicates (mean \pm SD). (B). The polyclonal T cells were stimulated overnight by a serial dilution of RdRp₈₂₉₋₈₃₇ and RdRp₈₂₉₋₈₃₈ respectively. The supernatants were taken for quantification of IFN- γ in ELISA assay. IFN- γ production depicted as mean values of three replicates (mean \pm SD). (C). FACS analysis of T cells activation markers 4-1BB and OX40 after overnight co-incubation of CD8⁺TCR1-T, CD8⁺TCR2-T and CD8⁺Con-T cells with COS-7-B*51:01-RdRp cells and COS-7-RdRp cells. (D). FACS analysis of T cells activation markers 4-1BB and OX40 after overnight co-incubation of CD4⁺TCR1-T, CD4⁺TCR2-T and CD4⁺Con-T cells with COS-7-B*51:01-RdRp cells and COS-7-RdRp cells.

activities of polyclonal T cells. In accordance with the higher functional affinity towards RdRp₈₂₉₋₈₃₇ demonstrated by polyclonal T cells, both TCR1-T and TCR2-T cells also showed more potent reactivities with RdRp₈₂₉₋₈₃₇ than RdRp₈₂₉₋₈₃₈ in the ELISA and FACS analyses. This is in line with the predicted ten times higher binding affinity of RdRp₈₂₉₋₈₃₇ with B*51:01 than RdRp₈₂₉₋₈₃₈ [24]. These results suggest that RdRp₈₂₉₋₈₃₇ is the core epitope unit.

SARS-CoV-2 specific CD8⁺ T cells are critical for the control of viral replication [25]. The results of ELISA, FACS analysis, and ELISPOT assay suggested that both the TCR-CD8⁺ T cells can specifically recognize the RdRp₈₂₉₋₈₃₇ epitope loaded onto B*51:01. Peptide concentration-dependent recognition was observed in the peptide dilution assay. The two TCRs exhibited similar functional avidity. By transducing the RdRp protein to the COS-7-B*51:01 cells, the recognition of intracellularly presented epitopes by both TCR-CD8⁺ T cells was further confirmed. Similarly, in both assays, the proportions of CD8⁺TCR⁺4-1BB⁺ T cells were significantly upregulated, verifying the TCR-HLA-mediated T cell activation [26,27]. In accordance with the above results, the proportion of IFN- γ ⁺TCR⁺CD8⁺ T cells was also upregulated, as detected by intracellular staining. When SARS-CoV-2 specific CD8⁺ T cells recognize the epitopes presented by virus-infected cells or APCs, they activate and differentiate to exhibit cytotoxic effects. CD8⁺ T cells can express higher levels of cytotoxic molecules, such as granzyme A and FAS ligand, which help eliminate virus-infected cells [9,28]. CD107a has been reported to be a cytotoxicity marker of T cells [23]. The significant up-regulation of CD8⁺TCR⁺CD107a⁺ cells verify the potential TCR-mediated specific cytotoxic effects.

Similar to CD8⁺ T cells, ELISA, FACS analysis, and ELISPOT assay confirmed the specific recognition of the RdRp₈₂₉₋₈₃₇ epitope by the two TCR-CD4⁺ T cells when loaded onto B*51:01. The blocking of such recognition by the anti-HLA-I antibody further verifies the HLA-TCR-mediated recognition. Peptide concentration-dependent recognition of TCR-CD4⁺ T cells was also observed in the peptide dilution assay. Like CD8⁺ T cells, the recognition of intracellularly presented epitopes by

both TCR-CD4⁺ T cells was also confirmed. In both assays, the proportions of CD4⁺TCR⁺4-1BB⁺ and CD4⁺TCR⁺OX40⁺ T cells were upregulated, verifying the TCR-HLA-mediated T cell activation [26,27]. The upregulated IFN- γ ⁺TCR⁺CD4⁺ T cells further verified the TCR-mediated recognition of intracellularly presented epitopes. Interestingly, the cytotoxicity marker CD107a of TCR⁺CD4⁺ T cells was also upregulated upon specific recognition. This suggests that the two CD8⁺ T cell-derived TCRs can also render CD4⁺ T cells the capabilities of epitope-specific cytotoxicity, highlighting the unique advantages of TCR-based therapies. While the cytotoxic functions are commonly dominated by CD8⁺ T cells, CD4⁺ T cells with cytotoxic potential are reportedly associated with better clinical outcomes in several viral infections in humans [29–32].

The antiviral function of SARS-CoV-2 specific T cells was further confirmed by measuring a panel of secreted cytokines. Upon specific activation, antiviral cytokines, including IFN- γ , IL-2, and IL-8, are secreted by both CD8⁺ and CD4⁺ TCR-T cells. Interestingly, some amount of IL-13 is also secreted by CD8⁺ TCR-T cells and CD4⁺ TCR2-T cells. IL-13 downregulates the activity of macrophages and limits neutrophil aggregation in the target organ to suppress inflammatory mediators during infection [33,34]. Macrophages and neutrophils are correlated with severe COVID-19 cases [9]. Therefore, IL-13 may help prevent severe tissue damage caused by inflammatory reactions as well as a cytokine storm. In addition, the anti-inflammatory cytokine IL-10 was uniquely secreted by the CD4⁺ TCR T-cells. IL-10 can also inhibit macrophage activity [35]. Moreover, IL-10 can ameliorate the excessive Th1 and CD8⁺ T cell responses responsible for much of the immunopathology associated with the infections [9,35]. Therefore, the combination of inflammatory and anti-inflammatory cytokines secreted by CD8⁺ and CD4⁺ T cells may be critical for the proper disease control of COVID-19.

The currently dominant SARS-CoV-2 variant, Omicron, renders most of the receptor-binding motif-targeted monoclonal antibodies lost in neutralizing activity [5,6]. TCR-T therapies targeting epitopes

conserved in SARS-CoV-2 variants and other coronaviruses may contribute uniquely to control the ongoing pandemic. Many attempts have been made to develop monoclonal antibodies targeting epitopes conserved in the subgenus Sarbecovirus [6,36,37]. In comparison, the targeted epitopes of TCR-T therapies can be highly conserved over a much broader range. The RdRp₈₂₉₋₈₃₇ epitope is not only strictly conserved in subgenus Sarbecovirus, including Omicron, but is also highly conserved in the four different genera of the subfamily *Coronavirinae*. Moreover, the four mutant epitopes from the *Coronavirinae* subfamily can also be recognized by the two TCRs. Interestingly, all mutated amino acids shared high similarities with the original amino acids. First, three of the four mutant epitopes identified from *Coronavirinae* possess a mutation at position P6 (Proline) (LPYPDPSRI). Like proline, the mutated amino acids at this position (alanine and valine) are also non-polar with hydrophobic side chains. The same phenomenon was observed for the mutation of L1 (leucine) to tyrosine. Moreover, the mutated amino acid at position D5 (aspartic acid) was found to be asparagine, which shares many structural and conformational similarities with aspartic acid. The similarities between mutations and original amino acids may partly explain the reserved recognitions by TCR-T cells. This also implies the essential involvement of this motif in the function of RdRp. Such superior conservativeness offers the great potential of conserved motifs to prevent the virus from becoming resistant to the TCR-T cell therapies, ensuring their efficacy in targeting the current and future SARS-CoV-2 variants.

Efficient availability is one of the concerns regarding TCR-T cell therapy. Such limitations can now be resolved using off-the-shelf allogeneic T cells [38]. In addition, HLA-B*51:01 is one of the most frequent HLA-B alleles and can cover approximately 5–10 % of the world population. Research to identify and evaluate TCRs targeting other highly frequent HLA-I alleles, including A*11:01, A*24:02, C*01:02, etc. is underway. With a combination of different allogeneic TCR-T cells in stock that can cover most populations, appropriate TCR-T cells can be chosen and transfused to high-risk populations after efficient HLA typing. The diversity of SARS-CoV-2 specific T cell responses has been reported to be associated with mild symptoms of COVID-19 [39]. Considering most current vaccines only induce immune responses against structural proteins, the transduced TCR-T cells targeting RdRp and other non-structural proteins can increase the diversity of circulating SARS-CoV-2 specific T cells for the vaccinated population. As reported, the continuous proliferation and dynamic differentiation of stem memory T cells into effector memory CD8⁺ T cells occurs in the stage of viral clearance and inflammation resolution of COVID-19 [25]. When needed, the matched off-the-shelf TCR-T cells can be transduced to the high-risk populations even before infection to allow the reservoir establishment of SARS-CoV-2 specific stem memory T cells. T cell therapy consisting of multiple viruses-specific T cell lines expanded from healthy seropositive donors has been shown to be safe and effective against infection of Epstein-Barr virus, adenovirus, cytomegalovirus, BK virus, and human herpesvirus 6 in allogeneic stem cell or solid organ transplants [40]. However, T cell therapies are often accompanied with cytokine release syndromes (CRS) which are also characteristic for acute severe COVID-19.

In vivo models by SARS-CoV-2 infecting humanized mice to evaluate the safety and efficacy profile of these TCR-T cells will be conducted. After which, for safety reasons the pilot clinical studies of this TCR-T therapy will be firstly tested in healthy subjects at high risk of SARS-CoV-2 infection, and COVID-19 patients with mild symptoms in which TCR-T cells may be helpful to prevent them from severe diseases. However, CRS may occur when it is used to treat COVID-19 patients with severe illness. While the RdRp-specific memory T cells may be pre-existed in infected population [41], the number of these T cells can be too few to be of great significance for the control of virus infection. The good efficacy profile of the two identified TCRs may suggest that the tested RdRp epitope can be a good vaccine target. Healthy individuals may also benefit from a targeted vaccine based on the tested epitope.

In summary, rapid identification and functional evaluations of TCRs targeting a pan-genera-conserved RdRp epitope highlight the unique potential of TCR-T cell therapies in controlling the ongoing pandemic, as well as in preparing for the next coronavirus outbreak [16].

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Ethical Approval statement

The PBMC were isolated from buffy coats obtained from healthy donors who had signed informed consent. This study was approved (20201130003-FS01) by the institutional review board of the Second People's Hospital of Shenzhen, Shenzhen, China.

CRediT authorship contribution statement

Yipeng Ma: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Fenglan Liu:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Bin Li:** Investigation, Methodology, Project administration. **Kaiqi Peng:** Investigation, Methodology, Project administration. **Hong Zhou:** Formal analysis, Investigation, Methodology, Visualization. **You Xu:** . **Dongjuan Qiao:** Formal analysis, Investigation, Methodology, Visualization. **Lijuan Deng:** Formal analysis, Investigation, Methodology, Visualization. **Geng Tian:** Resources, Investigation, Methodology. **Morten Nielsen:** Visualization. **Mingjun Wang:** Funding acquisition, Supervision, Validation, Visualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.intimp.2022.109283>.

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